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13. ABSTRACT ***(Maximum 200 Words)***

During the second year of the funded period, we focused on the characterization of interactions between hRAD51 and the five hRAD51 paralogs as well as interactions between these proteins and BRCA2, the BRCA1-interacting protein BARD1 and RPA. We detected strong interactions suggesting a stable complex, and weaker interactions. Some of these weaker interaction signals between hRAD51 paralogs increased in the presence of ATP and decreased in the presence of ADP which may indicate a regulatory role for adenosine nucleotides. Examination of the functional significance of these interactions is currently in progress.

We have also examined the role of hRAD51-dependent DNA repair by homologous recombination in BCR/ABL-expressing cells. We found that the oncogenic tyrosine kinase BCR/ABL upregulates hRAD51 and several hRAD51 paralogs. Elevated DNA repair by recombination seems to be a major pathway by which BCR/ABL-expressing cells become drug resistant. These findings may have significant implications for cancer therapy (see accompanying reprint Slupianek *at al.*, Mol.Cell 8, 2001).

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(4) INTRODUCTION:

DNA repair is essential for genomic integrity, and failure of repair pathways may lead to a mutator phenotype and to tumorigenesis (1, 2). Homologous DNA recombination (HR) is a prominent pathway for the repair of double-strand break (DSB) and other DNA lesions and is dependent on human RAD52 epistasis group proteins including hRAD51 and its five paralogs (3-6). Evidence for the involvement of both BRCA1 and BRCA2 in hRAD51-mediated repair processes is accumulating (7-9). The purpose of this study is to characterize physical and functional interactions among hRAD51 paralogs and BRCA1/BRCA2 in order to better understand HR.

(5) BODY

Brief summary of previously reported work

Aim I: done, results have been reported in Cancer Res. (Ref.11). The coding region of the hRAD51 gene has been examined for mutations in tumor tissues with high frequencies of 15q15 deletions, and the promoter region has been tested for hypermethylation. No changes have been found in the tumors compared to normal tissues.

Aim II: partially done, results have been reported. All known human RAD51 homologs involved in mitotic recombination have been cloned into appropriate vectors and overexpressed in bacteria or in a baculovirus system. Polyclonal antibodies against these proteins have been generated and characterized. Previously unknown chromosomal locations of RAD51 homologs have been determined. Purification of XRCC2 has been reported.

Summary of current work

Aim III a) Characterization of interactions between hRAD51 and its homologs. During the second year of the funded period, we focused on the characterization of interactions between hRAD51 and its human homologs. We had cloned members of the human RAD52 epistasis group into appropriate expression vectors: hRAD51, hRAD51B (a.k.a. hRAD51L1 or hRAD51-H2), hRAD51C (a.k.a. hRAD51L2), hRAD51D (a.k.a. hRAD51-H3, hRAD51L3), hXRCC2, hXRCC3 as well as hRAD52 and hRAD54. This collection allowed us to clearly identify static interactions between these six human mitotic RecA homologs, hRAD52, hRAD54 as detailed in Figures 1-7 (Aim IIIa). Interaction studies were done using an *in vitro* GST-fusion-IVTT method as described (10, 11). In this assay, hRAD51 gave a strong signal with itself as expected (Figure 1). We also found (weaker) interactions with hRAD51B, hRAD51D, hXRCC3, and hRAD52 (Figures 1-7). hRAD51B showed strong interactions with hRAD51C and hRAD51D and weaker signals with hXRCC3 (Figures 1-7). hRAD51C interacted strongly with hRAD51B and hRAD51D and less strongly with XRCC3 (Figures 1-7). Interestingly, hRAD51D seems also to be able to self-interact (Figure 4). hRAD51D interactions were detected with all other hRAD51 homologs (Figures 1-7). The strongest signal was seen with hXRCC2 (Figure 4 and 5), and hXRCC2 seems to interact only with hRAD51D (Figure 5). hXRCC3 also seems to be able to interact with all hRAD51 homologs (Figure 6), but seems to bind best to hRAD51D. In addition, we detected signals in the hRAD52

and hRAD54 lanes (Figure 6). hRAD52 bound to hRAD51, hRAD51B, hRAD51D, hXRCC3 and itself.

Since all RAD51 homologs have highly conserved ATP binding domains (Walker boxes) and ATP binding/hydrolysis seems to have a crucial role during HR (12), we further investigated whether interactions between the hRAD51 homologs could be modified by adenosine-nucleotide. We introduced point mutations in the ATP binding domain which can abolish nucleotide binding (K to A mutants) or ATP hydrolysis (K to R mutants) (13). Preliminary data show that binding of hRAD51 to the hXRCC3-GST fusion protein is dramatically reduced in the presence of ADP (Figure 8, right panel). In contrast, binding to hXRCC3 is much increased in the presence of ATP when hydrolysis is prevented by a K133R mutation (Figure 8). Similarly, the presence of ATP seems to increase binding of hRAD51D to hRAD51-GST, while interaction of the hRAD51D(K113A) mutant is much diminished (Figure 8, left panel). In addition, a hRAD51D splice variant (RDs1) with a deletion of exon 5 (exon 5 contains the Walker A box) has no significant binding activity to hRAD51-GST. These data indicate a regulatory role of ATP hydrolysis and/or binding. We currently perform experiments using non-hydrolysable ATP analogs to further confirm these findings.

In addition, co-immunoprecipitation experiments using specific antibodies and HeLa cell extracts are currently in progress.

Aim III b) Purification and characterization of hRAD51 derivatives. Purification and biochemical studies to characterize the human RAD51 paralogs are currently in progress.

Aim IV a) Interactions of hRAD51 derivatives with BRCA2, BARD1, and RPA (Aim IV). In order to test BRCA2 in our GST interaction assay, it was necessary to divide the 10.3kb BRCA2 ORF into four overlapping fragments of approximately 3kb each since we could not generate a full length labeled BRCA2 IVTT protein in sufficient quantities. While we have not tested the N-terminal fragment yet, the three other fragments seem to show significant interactions with several of the hRAD51 homologs (Figure 9). We have also cloned and tested the BRCA1-interacting protein BARD1. We found a very strong interaction signal in the hRAD52-GST lane (Figure 10) while the hRAD51 homologs show only a very weak signal. Finally, we obtained a strong interaction signal for the 70kDa subunit of hRPA(IVTT) with hRAD52-GST and weaker signals with the smaller hRPA subunits (Fig.11). These and the BRCA2 data have to be confirmed in reciprocal experiments with BRCA2-GST and BARD1-GST constructs, respectively.

Figure 12 summarizes the interactions found in the GST assay. This figure does not indicate that a stable complex like this exists in the cell, however, and our interaction experiments using adenosine nucleotides (see above) and other preliminary data rather suggest a more complex behavior of these proteins.

Characterization of hRAD51 and paralogs in BCR/ABL-mediated drug resistance (see accompanying reprint). In collaboration with Dr.T. Skorski (Temple University), we tested whether hRAD51-dependent HR is involved in drug resistance observed in cells

from chronic myelogenic leukemia (CML) patients and patients suffering from acute lymphocytic leukemia (ALL). These cells express BCR/ABL, a fusion protein generated by the translocation of chromosomes 9 and 22. We found that hRAD51 and several hRAD51 paralogs were upregulated in these cells. In addition, hRAD51 was phosphorylated by BCR/ABL. Increased DNA repair mediated by hRAD51-dependend HR seems to be a major mechanism by which BCR/ABL-expressing cells become drug resistant (14).

(6) KEY RESEARCH ACCOMPLISHMENTS:

- Interactions between hRAD51 and its homologs have been determined (Figures 1-7; Aim IIIa).

- Interactions between hRAD51 homologs seem to be modified in the presence of ATP or ADP (Figure 8). These findings may indicate a regulatory role for adenosine nucleotides during HR.

- Initial experiments have been done to test interactions between hRAD51 homologs and BRCA2, BARD1 and RPA (Figures 9-11; Aim IV). Significant interactions have been detected.

- A pathway has been described by which BCR/ABL-expressing cells increase DNA repair by hRAD51-dependent recombination leading to drug resistance (14).

(7) REPORTABLE OUTCOMES

All findings listed under (6) are reportable. Reprints describing the involvement of hRAD51 and paralogs in drug resistance in BCR/ABL cells are attached. Data describing interactions of hRAD51 and paralogs have been presented at the Keystone meeting "Molecular Mechanisms of DNA Replication and Recombination" in Snowbird, Utah. A manuscript is in preparation.

(8) CONCLUSIONS

- Multiple interactions exist between hRAD51 and its homologs and BRCA2 (Figures 1-9). Purification of these proteins is in progress in order to study the functional relevance of these interactions.

- DNA repair by hRAD51-dependent recombination is a major pathway for mediating drug resistance in BCR/ABL-expressing cells. It is possible that HR

may be upregulated in other cell types including breast cancer cells, which would have significant implication for cancer therapy.

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(10) APPENDICES

Figure 1: Interactions of human RAD51 homologs with hRAD51-GST. 35S-labeled full length hRAD51 and five human homologs were made by in vitro transcription-translation (IVTT) reaction and precipitated with the hRAD51-GST-fusion protein bound to glutathione beads as described (10, 11). As a control for non-specific binding to the beads, the IVTT proteins were incubated with GST-bound beads alone. After extensive washing, the proteins were eluted from the beads, separeated by SDS-PAGE and visualized by autoradiography.

Figure 2: Interactions of human RAD51 homologs with hRAD51B-GST.

Figure 3: Interactions of human RAD51 homologs with hRAD51C-GST.

Figure 4: Interactions of human RAD51 homologs with hRAD51D-GST.

Figure 5: Interactions of human RAD51 homologs with hXRCC2-GST.

Figure 6: Interactions of human RAD51 homologs with hXRCC3-GST.

Figure 7: Interactions of human RAD51 homologs with hRAD52-GST.

Figure 8: Left panel: Interactions of wild type hRAD51D and mutant hRAD51D with hRAD51-GST (RD: hRAD51D; RD-KR: hRAD51D(K113R); RD-KA: hRAD51D(K113A); RD+ATP: hRAD51D+1mM ATP; RD+ADP: hRAD51D+1mM ADP; RDs1: hRAD51D, exon 5 and 6 deleted). Right panel: Interactions of wild type hRAD51 and mutant hRAD51 with hXRRCC3-GST (R51: hRAD51; R51-KR: hRAD51(K133R); R51-KA: hRAD51(K133A); R51+ATP: hRAD51+1mM ATP; R51+ADP: hRAD51+1mM ADP)

Figure 9: Interactions of human RAD51 homologs and BARD1 with BRCA2-GST. BRCA2-partB: amino acids 1-998; BRCA2-partC: amino acids 927-1964; BRCA2-partD: amino acids 2581-3418(stop).

Figure 10: Interactions of human RAD51 homologs with BARD1-GST.

Figure 11: Interactions of human RAD51 homologs with hRPA-GST.

Figure 12: Interaction summary.

Reprints of: Slupianek *et al.*, Mol Cell 8, 795-806, 2001.

Note:

We have erred in not acknowledging the DOD as a source of funding for the studies reported in Slupianek, et al., There are two publications in preparation that will include this source of funding.

Fig.1

	hRAD51		hRAD51B		hRAD51C		hRAD51D	
hRAD51-GST	+		+		+		+	
GST		+		+		+		+

	XRCC2		XRCC3		hRAD52		hRAD54	
hRAD51-GST	+		+		+		+	
GST		+		+		+		+

Fig.2

	hRAD51		hRAD51B		hRAD51C		hRAD51D	
hRAD51B-GST	+		+		+		+	
GST		+		+		+		+

	XRCC2		XRCC3		hRAD52		hRAD54	
hRAD51B-GST	+		+		+		+	
GST		+		+		+		+

Fig.3

	hRAD51		hRAD51B		hRAD51C		hRAD51D	
hRAD51C-GST	+		+		+		+	
GST		+		+		+		+

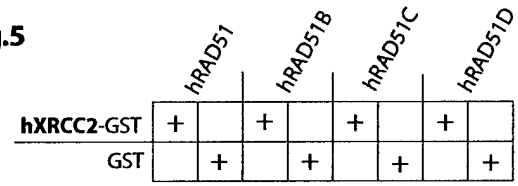
	XRCC2		XRCC3		hRAD52		hRAD54	
hRAD51C-GST	+		+		+		+	
GST		+		+		+		+

Fig.4

	hRAD51		hRAD51B		hRAD51C		hRAD51D	
hRAD51D-GST	+		+		+		+	
GST		+		+		+		+

	XRCC2		XRCC3		hRAD52		hRAD54	
hRAD51D-GST	+		+		+		+	
GST		+		+		+		+

Fig.5



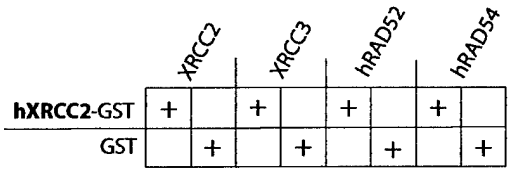
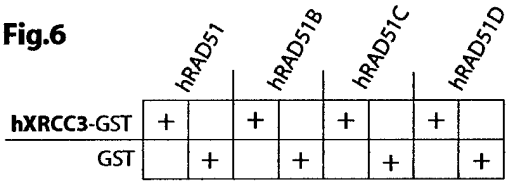
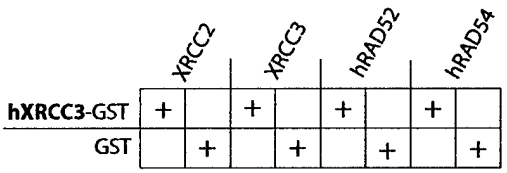


Fig.6





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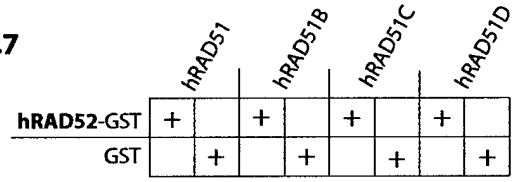

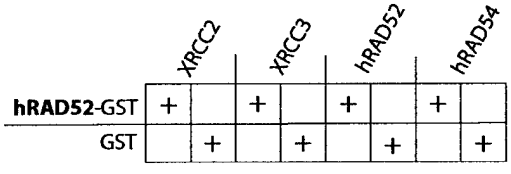

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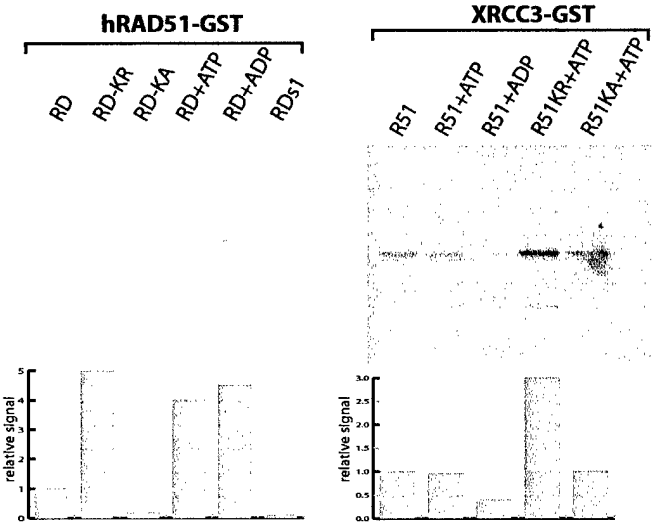

RD: hRAD51D
RD-KR: hRAD51D(K113R)
RD-KA: hRAD51D(K113A)
RD+ATP: hRAD51D+ 1mM ATP
RD+ADP: hRAD51D+1mMADP
RDs1: hRAD51D(del-exon5+6)

R51: hRAD51
R51-KR: hRAD51(K133R)
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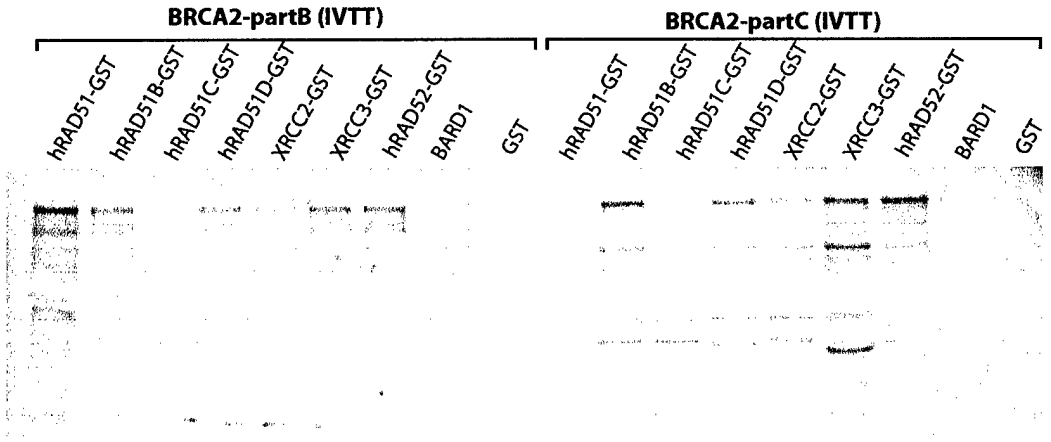
R51+ATP: hRAD51+ 1mM ATP
R51+ADP: hRAD51+1mMADP

Fig.9



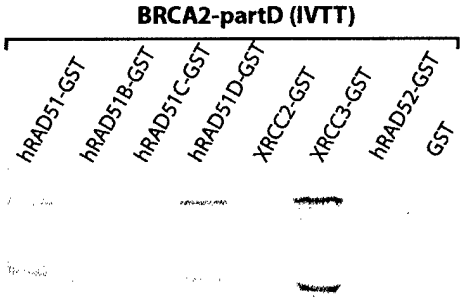
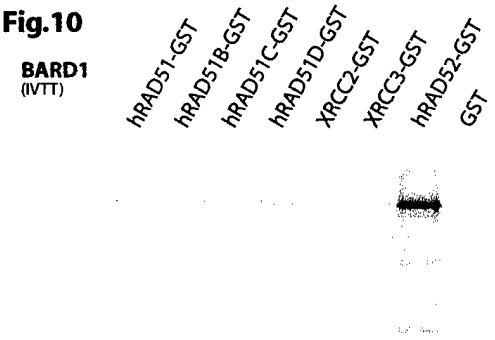


Fig.10



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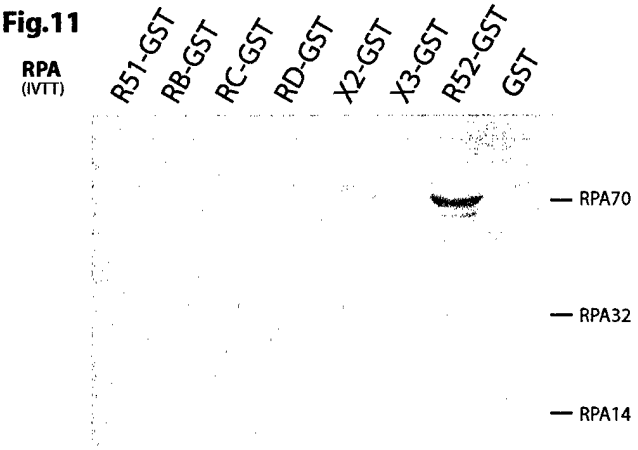
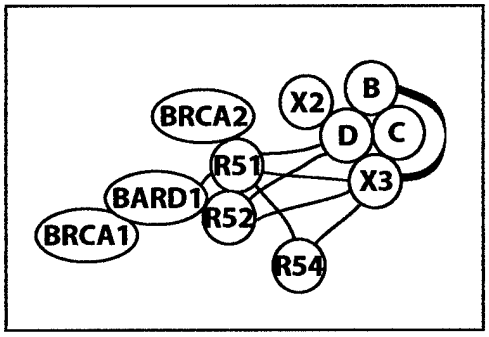


Fig.12



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BCR/ABL Regulates Mammalian RecA Homologs, Resulting in Drug Resistance

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Summary

RAD51 is one of six mitotic human homologs of the *E. coli* RecA protein (RAD51-Paralogs) that play a central role in homologous recombination and repair of DNA double-strand breaks (DSBs). Here we demonstrate that RAD51 is important for resistance to cisplatin and mitomycin C in cells expressing the BCR/ABL oncogenic tyrosine kinase. BCR/ABL significantly enhances the expression of RAD51 and several RAD51-Paralogs. RAD51 overexpression is mediated by a STAT5-dependent transcription as well as by inhibition of caspase-3-dependent cleavage. Phosphorylation of the RAD51 Tyr-315 residue by BCR/ABL appears essential for enhanced DSB repair and drug resistance. Induction of the mammalian RecA homologs establishes a unique mechanism for DNA damage resistance in mammalian cells transformed by an oncogenic tyrosine kinase.

Introduction

Tumors expressing oncogenic tyrosine kinases such as BCR/ABL, v-SRC, and HER-2/neu are relatively resistant to DNA damage induced by therapeutic drugs (Bedi et al., 1995; Masumoto et al., 1999; Nishii et al., 1996; Pietras et al., 1994). For BCR/ABL tumors, drug resistance does not appear to be a result of clonal selection and depends directly on the kinase activity of the oncogenic protein (Nishii et al., 1996). The BCR/ABL fusion is derived from translocation of the c-ABL gene from chromosome 9 to the BCR gene locus on chromosome 22 [Philadelphia chromosome, t(9;22)] and is present in most chronic myelogenous leukemia (CML) patients as well as a cohort of acute lymphocytic leukemia (ALL) patients (Clark et al., 1988; Shtivelman et al., 1986). The BCR/ABL translocation produces p230, p210, or p185 fusion proteins, all of which exhibit constitutive tyrosine kinase activity and cause CML- or ALL-like syndromes in mouse models (Li et al., 1999).

Several mechanisms have been proposed that may contribute to the drug resistance associated with neoplastic transformation (for review see el-Deiry, 1997). However, there has been no comprehensive examination of DNA repair processes which might significantly contribute to the removal of therapeutic drug- and/or irradiation-induced DNA damage. Our pilot studies suggested that signaling from the BCR/ABL *s*rc *h*omology-3 (SH3) and *s*rc *h*omology-2 (SH2) domains induced a drug-resistant phenotype which was dependent on the activation of *s*ignal *t*ransducer and *a*ctivator of *t*ranscription 5 (STAT5). We subsequently identified RAD51 as an elevated transcript associated with STAT5 activation in BCR/ABL cells. RAD51 has been proposed to play a central role in the repair of DNA *d*ouble-strand *b*reaks (DSBs) via its catalysis of homologous recombination between sister chromosomes/chromatids (Shinohara and Ogawa, 1995). Incorrect repair of DSBs may lead to chromosomal loss or translocation, while unrepaired DSBs lead to programmed cell death (for review see Flores-Rozas and Kolodner, 2000).

Here we show that activation of the human oncogenic tyrosine kinase BCR/ABL controls the transcription, degradation, and phosphorylation of RAD51. We also demonstrate the altered expression of other mitotic RecA homologs (RAD51-Paralog proteins). BCR/ABL-mediated regulation of RAD51 and RAD51-Paralog proteins components results in increased recombination repair, which correlates with cisplatin and mitomycin C drug resistance in leukemia cells. These studies connect BCR/ABL (and perhaps other oncogenic tyrosine kinases) to DNA repair.

Results

BCR/ABL Induces Drug Resistance and Elevates the Expression of RAD51

The expression of BCR/ABL in 32Dcl3 cells results in protection from apoptosis when growth factor is removed (Nieborowska-Skorska et al., 1999; Skorski et al., 1997) as well as resistance to the DNA crosslinking drug cisplatin and the radiomimetic drug mitomycin C (Figures 1A, 1B, and 1C). The resistance to these drugs was evident in a clonogenic assay (Figure 1A), a trypan-blue viability assay (Figure 1B), or a TUNEL assay of apoptotic cells (Figure 1C). These results demonstrate concordance of three independent measures for determining drug resistance and cellular survival following drug treatment.

To ascertain the BCR/ABL signaling pathway(s) responsible for drug resistance, we employed a BCR/ABL mutant strategy (Nieborowska-Skorska et al., 1999; Skorski et al., 1997). We found that cell lines expressing a BCR/ABLΔΔ mutant (BCR/ABLΔΔ, a double deletion which removes the SH3 + SH2 domains) largely regained sensitivity to both cisplatin and mitomycin C

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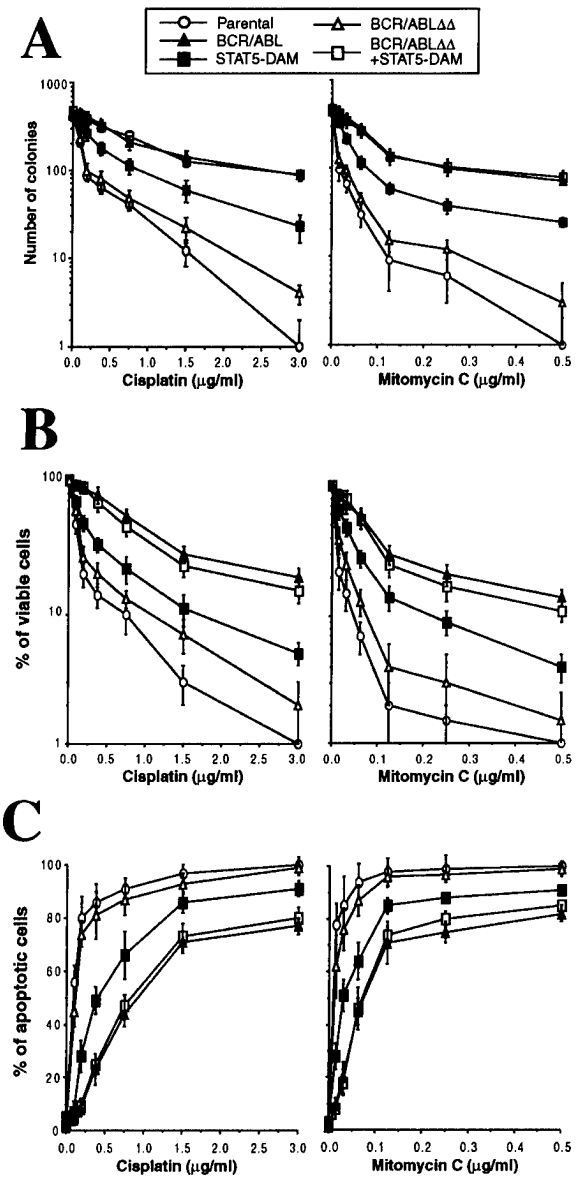

Figure 1. BCR/ABL SH3 + SH2→STAT5 Pathway Induces Drug Resistance

Following treatment with the therapeutic drugs cisplatin or mitomycin C, cells were evaluated for ([A]) clonogenic survival, ([B]) viable cells, or ([C]) apoptotic cells. Cells include 32Dcl3 cells (Parental) or 32Dcl3 cells expressing BCR/ABL (BCR/ABL), BCR/ABL ΔSH3 + ΔSH2 mutant (BCR/ABLΔΔ), STAT5B dominant-active mutant (STAT5-DAM), or BCR/ABL ΔSH3 + ΔSH2 mutant and STAT5B-DAM (BCR/ABLΔΔ+STAT5B-DAM). The average of three experiments (+/− SD) is shown.

(Figures 1A, 1B, and 1C). These results implicate signaling from BCR/ABL SH3 + SH2 domains in cisplatin and mitomycin C drug resistance.

The STAT5 transcription transactivator is an important downstream effector of BCR/ABL (for review see Danial and Rothman, 2000) that is not activated by the BCR/ABLΔΔ mutation (Nieborowska-Skorska et al., 1999). Coexpression of BCR/ABLΔΔ mutation and a STAT5B *d*ominant-*a*ctive *m*utant (STAT5-DAM) resulted in the full recovery of resistance to cisplatin and mitomycin C (Figures 1A, 1B, and 1C). These results should be compared to STAT5-DAM cells or BCR/ABLΔΔ cells, which appear to display an intermediate or weak drug resistance, respectively (Figures 1A, 1B, and 1C). Interestingly, the partial resistance displayed by parental cells expressing BCR/ABLΔΔ or STAT5-DAM appears additive to the drug resistance induced by cells expressing BCR/ABLΔΔ + STAT5-DAM (Figures 1A, 1B, and 1C). These results suggest that the SH3 + SH2 domain of BCR/ABL mediates resistance to cisplatin and mitomycin C through STAT5 activation.

The activation of STAT5 appeared to result in altered expression of a large number of genes (Mui et al., 1996). We identified a significant elevation of both the RAD51 mRNA and protein in BCR/ABL or BCR/ABLΔΔ + STAT5-DAM drug-resistant cells but not in the parental 32Dcl3 or BCR/ABLΔΔ drug-sensitive cells (Figure 2A). Furthermore, several myeloid and lymphoid cell lines, expressing different forms of BCR/ABL (p230, p210, or p185), also displayed elevated levels of RAD51 mRNA and protein (data not shown). RAD51 protein expression was elevated in primary mononuclear cells obtained from bone marrow samples of patients with chronic-phase CML (CML-CP) or blast-crisis CML (CML-BC) compared to normal healthy donors (NBMC), irrespective of the presence of growth factor (IL-3) (Figure 2B). Thus, overexpression of RAD51 is likely to be a common feature of human CML.

Cellular proliferation does not appear to correlate with the level of RAD51 expression. Parental 32Dcl3 cells and BCR/ABL cells display similar cell proliferation rates and cell cycle distribution in the presence of growth factor (IL-3) (data not shown). Moreover, highly proliferative CML cell lines expressing BCR/ABL (K562, BV173, EM-2) displayed elevated levels of RAD51, while an equally proliferative AML cell line that does not express BCR/ABL (HL60) displays significantly lower levels of RAD51 (data not shown).

Six homologs of the bacterial RecA and RAD51 proteins (RAD51-Paralogs) have been identified in mitotic human cells (hRAD51, hRAD51B, hRAD51C, hRAD51D, XRCC2, and XRCC3). Genetic and biochemical studies have demonstrated that these RAD51-Paralogs are not functionally redundant (Takata et al., 2001). These and other results have suggested that the RAD51-Paralogs act in concert to perform homologous recombination (HR). We examined the expression of the mitotic RAD51-Paralogs in primary mononuclear cells isolated from a CML-BC patient (Figure 2C). In addition to hRAD51, hRAD51B, hRAD51D, and hXRCC2 display elevated expression in CML-BC cells compared to NBMC. However, the expression of hRAD51C and hXRCC3 appeared to decrease in CML-BC cells compared to NBMC. While the exact role of individual mitotic RAD51-Paralogs in HR is unknown, it is clear that the overexpression of hRAD51 in BCR/ABL tumor cells is not unique. The altered expression of multiple RAD51-Paralogs suggests the RAD51-Pathway may be affected in BCR/ABL cells. It is interesting to note that other than hRAD51, only the hRAD51C-hXRCC3 heterodimer has been shown to perform recombinational strand exchange in vitro (Kurumizaka et al., 2001). Perhaps the coincident expression of hRAD51/hRAD51B/hRAD51D/hXRCC2 or hRAD51C/

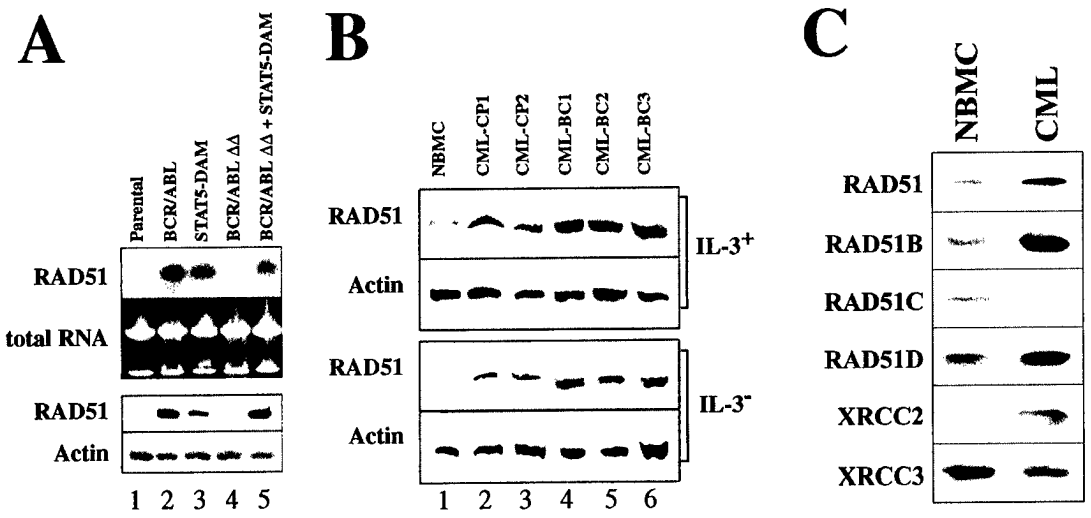

Figure 2. BCR/ABL Modulates the Expression of RAD51 and RAD51-Paralogs

(A) Total RNA or total cellular proteins were isolated from indicated IL-3-starved cells. RAD51 expression was examined by Northern analysis using total RNA as a loading control (upper panel) or Western analysis using actin as a loading control (lower panel).

(B) $CD34^+$ bone marrow mononuclear cells from two chronic myelogenous leukemia chronic phase (CML-CP) patients, three chronic myelogenous leukemia blast crisis (CML-BC) patients, and normal bone marrow cells (NBMC) from healthy volunteers were incubated for 12 hr in the presence (upper panel) or absence (lower panel) of human recombinant IL-3. Expression of RAD51 compared to actin was detected in total cell lysates by Western analysis.

(C) Cellular lysates from a CML-BC patient and NMBC were examined by Western analysis for the six known RecA homologs expressed in mitotic human cells.

hXRCC3 defines two separate recombination repair pathways.

RAD51 Protein Is Regulated by BCR/ABL-Dependent Activation of STAT5 and Prevention of Caspase-3 Activation

As a potent stimulator of transcription, STAT5 was examined for a role in the transactivation of the RAD51 promoter (Schmutte et al., 1999). Robust transactivation of the RAD51 promoter was detected in cells expressing BCR/ABL or mutant forms of BCR/ABL (SH3-P1013L + SH2-R1053L double point mutations or the ΔSH3 deletion mutant + SH2-R1053L point mutation) which retain the ability to stimulate STAT5 (Figure 3A; Nieborowska-Skorska et al., 1999). However, both the kinase-inactive BCR/ABL (K1172R) and the BCR/ABL mutations (ΔSH3 + ΔSH2 double-deletion mutation or the SH3-P1013L point mutation + ΔSH2 deletion mutation), which do not activate STAT5 (Nieborowska-Skorska et al., 1999), were incapable of transactivating expression from the RAD51 promoter (Figure 3A).

In addition to STAT5, signaling from the BCR/ABL SH3 + SH2 domains activates phosphotidylinositol-3 kinase (PI-3k), the Akt serine/threonine kinase, and the c-Myc transcriptional factor (Skorski et al., 1997). Transactivation of the RAD51 promoter by these other proteins was examined by a dominant-active/dominant-negative strategy. A dominant-active mutant (DAM) of STAT5 (STAT5-DAM) but not dominant-active mutants of PI-3k (PI-3k-DAM, p110*) or Akt (Akt-DAM, Akt E40K) exerted strong transactivation activity of the RAD51 promoter (Figure 3B). Conversely, in the presence of BCR/ABL the dominant-negative mutant (DNM) of STAT5 (STAT5-DNM, ΔSTAT5B) but not the other dominant-negative mutants (PI-3k-DNM, Δp85; Akt-DNM, K179M; c-Myc-DNM, In373) inhibited transactivation of the RAD51 promoter (Figure 3C). The role of STAT5 in IL-3-independent transcriptional regulation of RAD51 was confirmed by Northern blot analysis (Figure 3D). These results provide a strong and unique correlation between BCR/ABL-activated STAT5 and the overexpression of RAD51.

The RAD51 protein has been shown to be a substrate for caspase-3 (Huang et al., 1999). Expression of BCR/ABL has been shown to prevent activation of caspase-3 (Amarante-Mendes et al., 1998; Dubrez et al., 1998). To examine the role of caspase-3 in the regulation of RAD51 protein levels, parental 32Dcl3 cells, BCR/ABL cells, and BCR/ABLΔΔ cells were treated with cisplatin in the presence or absence of the caspase-3 inhibitor Z-DEVD-fmk. The activated caspase-3 fragments (19 kDa and 17 kDa) and caspase-3 cleaved RAD51 fragment (~23 kDa) was detected by Western analysis (Figure 3E). Activation of caspase-3 and the signature proteolytic degradation product of RAD51 only occurred in parental 32Dcl3 cells after treatment with cisplatin (Figure 3E, compare lanes 1 and 4). Addition of Z-DEVD-fmk to the cisplatin-treated parental 32Dcl3 cells resulted in the absence of the signature RAD51 proteolytic degradation product (Figure 3E, lane 5). BCR/ABL cells displayed neither caspase-3 activation nor RAD51 proteolytic degradation (Figure 3E, lanes 2 and 6). In the presence of cisplatin, the mutant BCR/ABLΔΔ displayed both caspase-3 activation and RAD51 degradation (Figure 3E, lane 7), which was largely inhibited by Z-DEVD-fmk (Figure 3E, lane 8). These results implicate BCR/ABL inhibition of caspase-3 activation, at least in part, for the increased levels of RAD51 protein. Taken to-

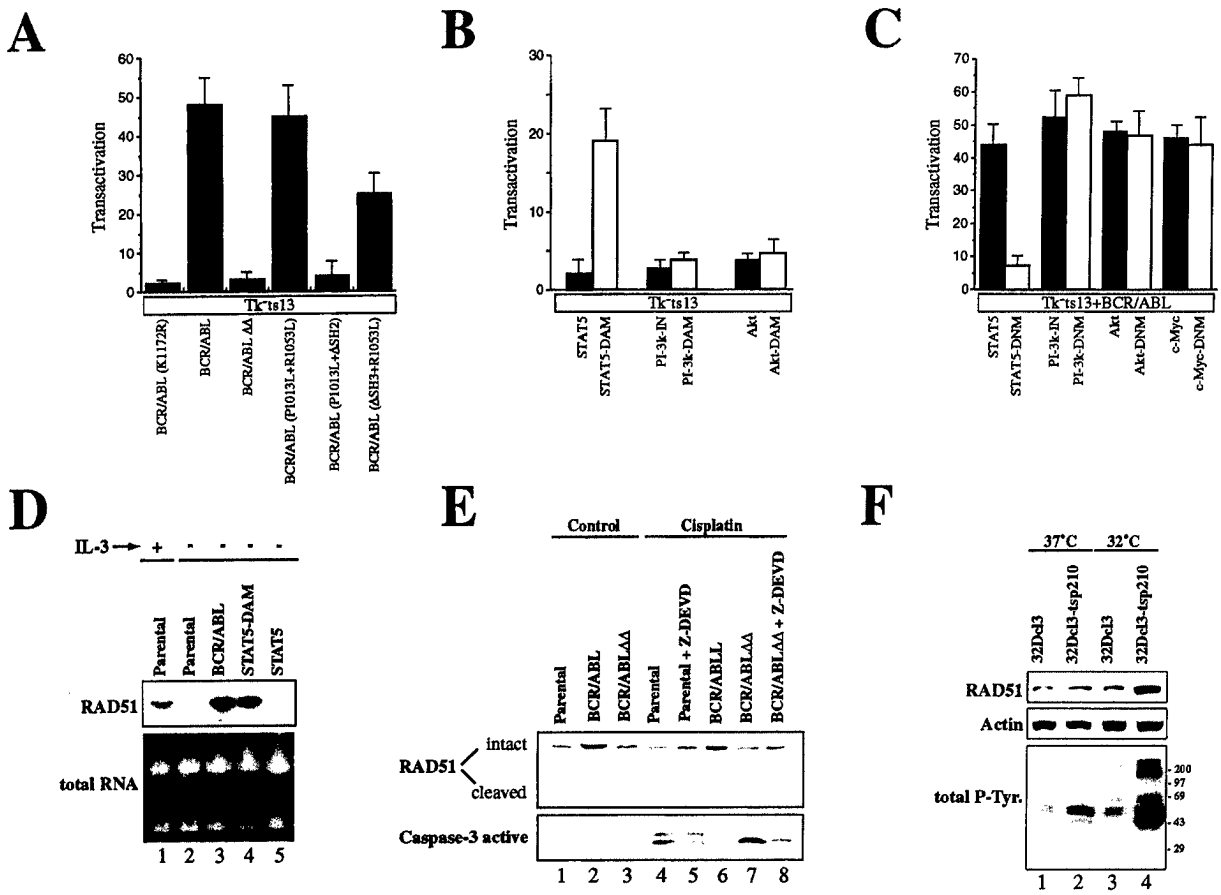

Figure 3. BCR/ABL Tyrosine Kinase Enhances Expression of RAD51 by Stimulation of STAT5-Mediated Transactivation and Inhibition of Caspase-3-Dependent Degradation

(A) The effect of BCR/ABL wild-type or indicated mutants.

(B) The effect of wild-type/inactive mutant (IN) STAT5B, PI-3k, or Akt (black bars) compared to dominant-active mutants (DAM) (open bars).

(C) The effect of STAT5, PI-3k, Akt, or c-Myc dominant-negative mutants (DNM) (open bars) compared to wild-type/inactive mutant (IN) (black bars). Results represent the average and standard deviation of three independent experiments.

(D) The role of STAT5 in RAD51 transactivation. Northern analysis of RAD51 from cell lines expressing indicated proteins.

(E) The role of caspase-3 in RAD51 protein degradation. Parental 32Dcl3 cells (Parental) and 32Dcl3 cells expressing BCR/ABL (BCR/ABL) or BCR/ABLΔSH3 + ΔSH2 mutant (BCR/ABLΔΔ) were untreated or treated with cisplatin (1 μg/ml) in the presence of IL-3 and Z-DEVD-fmk (50 μM) when indicated. RAD51 (full-length and cleaved form) and activated caspase-3 (19 kDa and 17 kDa fragments) were detected by Western analysis.

(F) The role of BCR/ABL kinase activity on RAD51 phosphorylation. Parental 32Dcl3 cells or 32Dcl3 cells expressing temperature-sensitive p210 BCR/ABL mutant (tsp210) were incubated for 24 hr at the permissive (32°C) or the restrictive (37°C) temperature in the presence of IL-3. Expression of RAD51 and the actin loading control was determined by Western analysis (upper panels). BCR/ABL total kinase activity (total-P-Tyr) was detected by Western analysis with anti-P-Tyr antibodies (lower panel).

gether, these observations are consistent with the BCR/ABL-dependent activation of STAT5 as well as the BCR/ABL-dependent inhibition of caspase-3 activation as a combined mechanism for the increased levels of RAD51 protein.

To address the possibility that clonal selection might account for the altered levels of RAD51, we examined a derivative of parental 32Dcl3 cells that stably expressed a temperature-sensitive mutant of p210-BCR/ABL kinase (tsp210). In these studies, the effect of BCR/ABL kinase activity on RAD51 protein levels could be determined in a dynamic unselected population of cells (Figure 3F). RAD51 protein expression and total cellular phosphorylated tyrosines (P-Tyr) were examined by Western analysis. In BCR/ABL tsp210 cells, elevated levels of RAD51 were observed at the permissive temperature (32°C) coincident with high levels of P-Tyr (Figure 3F, lane 4), compared to the restrictive temperature (37°C; Figure 3F, lane 2). These results correlate the kinase activity of BCR/ABL with an increase in RAD51 protein.

RAD51 Plays an Essential Role in BCR/ABL-Induced Cisplatin and Mitomycin C Drug Resistance

Because RAD51 overexpression appeared to closely correlate with drug resistance in BCR/ABL cells, we sought to determine its specific role in this process(es). BCR/ABLΔΔ cells were transduced with the RAD51 sense (S)-IRES-GFP retroviral vector to increase the expression of RAD51 comparable to BCR/ABL cells. Conversely, BCR/ABL cells were transduced with the RAD51 antisense (AS)-IRES-GFP retroviral vector to decrease

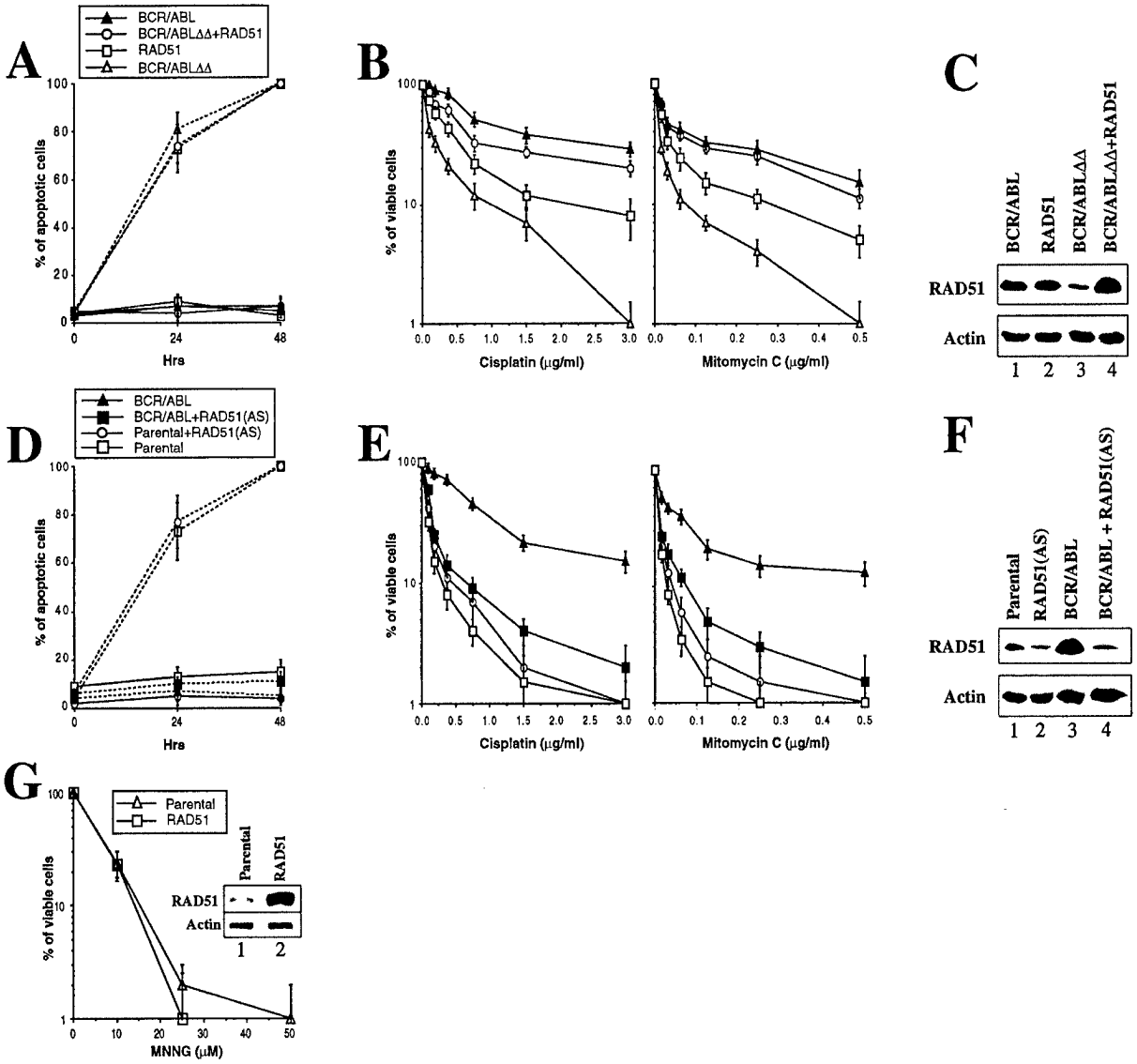

Figure 4. BCR/ABL-Induced Overexpression of RAD51 Is Essential for Inhibition of Drug-Induced Apoptosis

In (A)–(C), parental 32Dcl3 cells and BCR/ABLΔΔ cells were transduced with RAD51-IRES-GFP or IRES-GFP (see legend box above [A]). In (D)–(G), parental 32Dcl3 cells and BCR/ABL cells were transduced with RAD51(AS)-IRES-GFP or IRES-GFP (see legend box above [D]).

(A) The percentage of apoptotic cells. Cells were grown in the presence (solid lines) or absence (dashed lines) of IL-3.

(B) The percentage of viable (trypan blue-excluding) cells following treatment with cisplatin or mitomycin C.

(C) Western analysis of RAD51 in cells examined in (A) and (B) compared to actin loading control.

(D) The percentage of apoptotic cells grown in the presence (solid lines) or absence (dashed lines) of IL-3.

(E) The percentage of viable (trypan blue-excluding) cells following treatment with cisplatin or mitomycin C.

(F) Western analysis of RAD51 in cells examined in (D) and (E) compared to actin loading control.

(G) The percentage of viable (trypan blue-excluding) cells following treatment with MNNG. The inset contains Western analysis of RAD51 in cells examined in (G) compared to actin loading control.

the RAD51 expression comparable to 32Dcl3 parental cells. The GFP-positive cells were then isolated by fluorescence-activated cell sorter (FACS). Induced expression of RAD51 in BCR/ABLΔΔ cells (Figure 4C) rescued cisplatin and mitomycin C resistance to levels comparable with BCR/ABL cells (Figure 4B). Reciprocally, reduction of RAD51 expression in BCR/ABL cells to the levels observed in parental 32Dcl3 cells (Figure 4F) resulted in abrogation of cisplatin and mitomycin C resistance (Figure 4E). These results implicate high levels of RAD51 expression with drug resistance. However, the expression levels of RAD51 did not affect the susceptibility of cells to apoptosis induced by growth factor withdrawal (Figures 4A and 4D). For example, BCR/ABLΔΔ cells expressing high levels of RAD51 (Figure 4C, lane 4) remained susceptible to apoptosis induced by IL-3 growth factor withdrawal (Figure 4A, compare IL-3$^-$ dashed lines to IL-3$^+$ solid lines; Nieborowska-Skorska et al., 1999), while BCR/ABL cells expressing low levels of RAD51 (Figure 4F, lane 4) remained resistant to apo-

ptosis induced by IL-3 growth factor withdrawal (Figure 4D, compare IL-3$^-$ dashed lines to IL-3$^+$ solid lines). These results appear to separate the drug resistance induced by BCR/ABL-dependent overexpression of RAD51 from the well-described BCR/ABL-dependent resistance to apoptosis induced by growth factor withdrawal (McGahon et al., 1994). These results suggest that alteration of RAD51 protein levels selectively affects drug resistance.

Overexpression of RAD51 Does Not Affect MNNG Drug Resistance

DNA lesions are processed by a variety of repair pathways and repair mechanisms in eukaryotic cells. Alkylation damage appears to be processed by the base excision repair (BER), nucleotide excision repair (NER), and mismatch repair (MMR) pathways (for review see Fishel, 1999; Friedberg et al., 1995). To examine the specificity of RAD51-mediated drug resistance, we determined the effect of N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) on cells overexpressing RAD51 (Figure 4G). In spite of at least a 10-fold overexpression of RAD51 (compare lanes 1 and 2 of Figure 4G inset), we observed no resistance to the cytotoxic effects of MNNG (Figure 4G). These results are consistent with the notion that RAD51-induced drug resistance is lesion specific.

BCR/ABL-Dependent Overexpression of RAD51 Stimulates Homologous Recombination Repair of DSBs

The frequency of DSB repair can be measured in vivo by examining the formation of wild-type GFP resulting from the recombination of heteroallelic non-functional GFP fragments in Draa cells (Pierce et al., 1999). To test the effect of RAD51 levels on DSB repair, we stably introduced BCR/ABL (overexpression of RAD51) or the kinase-defective mutant BCR/ABL(K1172) (background expression of RAD51) (see Figures 2 and 3). We observed a significant increase in GFP recombination in BCR/ABL-Draa cells (Figure 5, lane 3) compared to parental Draa cells (Figure 5, lane 1) or Draa cells expressing BCR/ABL(K1172R) (Figure 5, lane 5). Reduction of the RAD51 levels in BCR/ABL cells by introduction of RAD51(AS) resulted in a significant reduction of GFP recombination (Figure 5, compare lane 4 with lane 3). The decrease in GFP recombination correlated with a decrease in the RAD51 protein levels (Figure 5, Western analysis). These results provide a link between RAD51-dependent homologous recombination repair and the kinase signaling functions of BCR/ABL.

BCR/ABL Interacts with RAD51

The cellular localization of RAD51 in parental and BCR/ABL-expressing cells was examined by immunofluorescence (Figure 6A). Consistent with the Northern and Western analysis (Figure 2A), BCR/ABL cells appear to express significantly more RAD51 than parental cells (Figure 6A). A punctate nuclear, perinuclear, and a diffuse cytoplasmic pattern of expression was observed for RAD51 protein in both the parental and BCR/ABL cells (Figure 6A, RAD51 column). The perinuclear and cytoplasmic localization of RAD51 was surprising and appears to contradict previous findings (Scully et al., 1997). However, recent studies have confirmed that

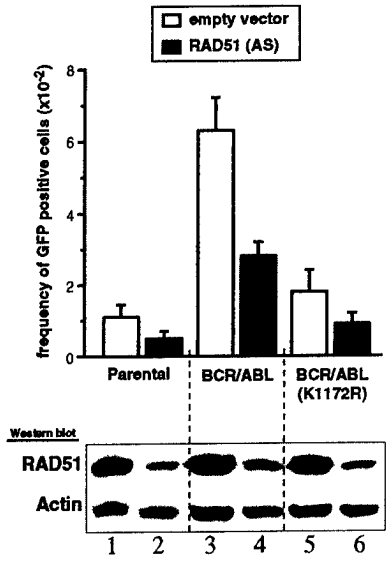

Figure 5. BCR/ABL Stimulates RAD51-Dependent Homologous Recombination Repair Activity of a Double-Strand Break

Draa recombination-reporter cells expressing BCR/ABL (BCR/ABL), the kinase-deficient mutant BCR/ABL K1172 R) or the *neo*-resistance vector alone (Parental). Stable cell lines were transfected with an I-SceI expression plasmid (to induce DSB within one of the heterozygous GFP alleles) along with either an empty expression vector (open bars) or the expression vector containing RAD51-antisense construct (RAD51 [AS]) (black bars). Homologous recombination repair of GFP alleles was evaluated after 48 hr by flow cytometry. Expression of RAD51 was determined by Western analysis compared to actin loading control (lower panel). Results represent the average and standard deviation of three independent experiments.

RAD51 is located in both the cytoplasm and nucleus in normal cells and is largely cytoplasmic in BRCA2-defective cells (Davies et al., 2001; Moynahan et al., 2001). BCR/ABL was largely confined to the perinuclear and cytoplasmic regions of the BCR/ABL-expressing cells (Figure 6A, BCR/ABL column). We observed a portion of the overexpressed RAD51 to overlap with BCR/ABL in the cytoplasm, suggesting a physical interaction between a subset of BCR/ABL and RAD51 proteins (Figure 6A, DAPI-RAD51-BCR/ABL merged panels).

DNA damage-dependent phosphorylation of RAD51 has been linked to a stable interaction between RAD51 and the c-ABL tyrosine kinase (Chen et al., 1999; Yuan et al., 1998). We performed coimmunoprecipitations from parental 32Dcl3 cells and isogenic BCR/ABL-expressing cells using a RAD51-specific antibody followed by Western analysis using an anti-c-ABL antibody. The c-ABL protein was observed in the parental 32Dcl3 cells, while both c-ABL and BCR/ABL protein were present in BCR/ABL cells (Figure 6B, lanes 3–6). The quantity of c-ABL and BCR/ABL proteins in these RAD51 immunoprecipitates did not appear to vary significantly following treatment with cisplatin and mitomycin C for 6 hr (Figure 6B, lanes 3–6). These results are consistent with the notion that at least some fraction of RAD51 forms a stable complex with both c-ABL and BCR/ABL.

The BCR/ABL Tyrosine Kinase Phosphorylates RAD51

Because of the intriguing colocalization of RAD51 and BCR/ABL, the phosphorylation status of RAD51 in pa-

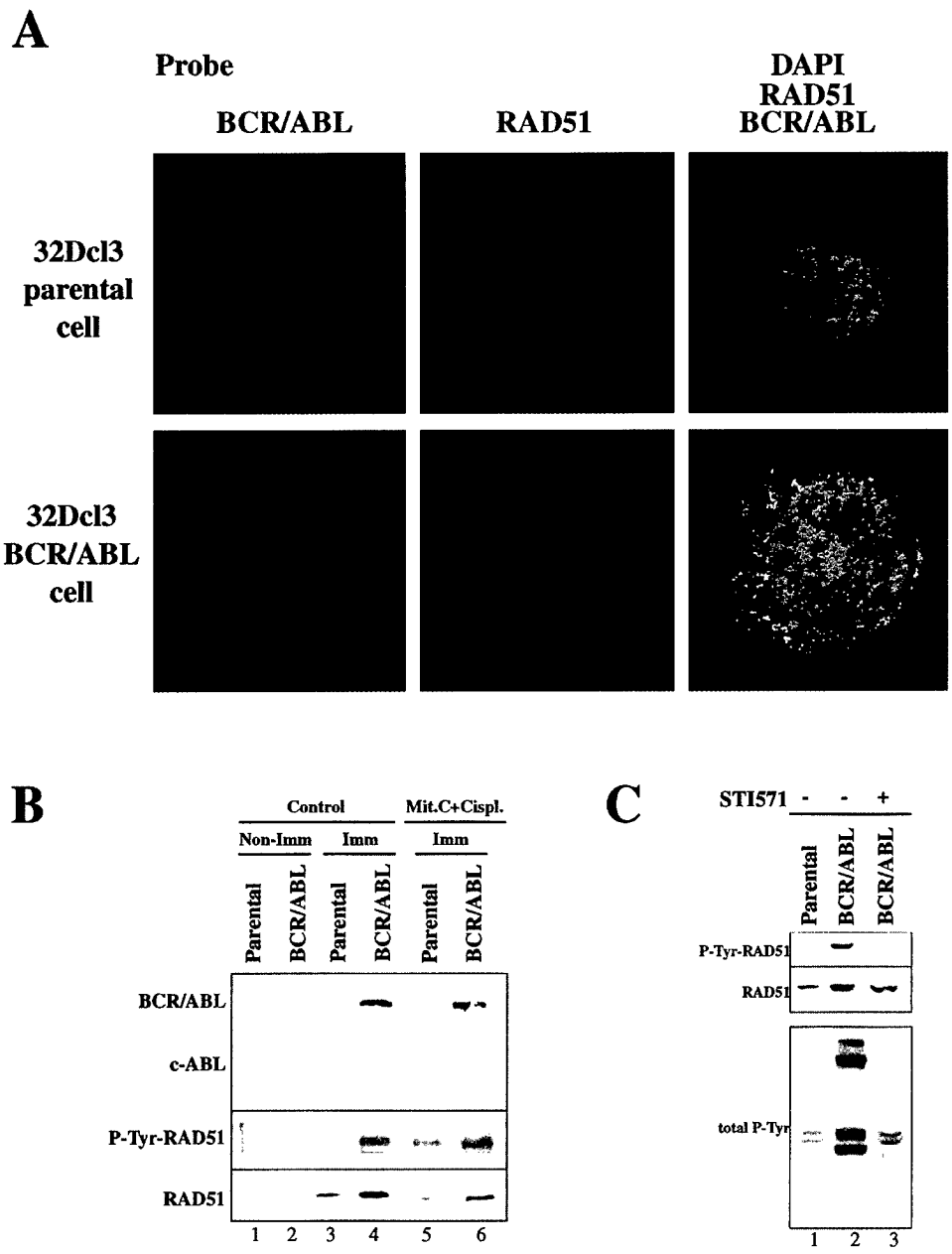

Figure 6. Interaction and Colocalization of BCR/ABL and RAD51

(A) Immunofluorescence microscopy of representative parental 32Dcl3 cells and BCR/ABL cells showing the localization of BCR/ABL (green) and RAD51 (red) proteins. Merged panels including DAPI stained nuclei are shown in right panels. Colocalization of BCR/ABL and RAD51 is indicated as yellow fluorescence.

(B) Coimmunoprecipitation of RAD51 with BCR/ABL. Parental 32Dcl3 cells (Parental) and BCR/ABL cells (BCR/ABL) were untreated (Control) or treated with mitomycin C (0.5 μg/ml) and cisplatin (3 μg/ml) (Mit.C+Cispl) for 6 hr in the presence of IL-3. The presence of ABL proteins (BCR/ABL and c-ABL) and RAD51 protein in anti-RAD51 immunoprecipitates was detected by Western analysis. Tyrosine phosphorylation of RAD51 (P-Tyr-RAD51) was determined using anti-phosphotyrosine antibodies. The results are representative of at least three independent experiments.

(C) The dependence of RAD51 tyrosine phosphorylation on BCR/ABL kinase activity. Parental 32Dcl3 cells (Parental) and BCR/ABL cells were incubated with (+) or without (−) STI571 (1 μM). Tyrosine phosphorylation of RAD51 (P-Tyr-RAD51; top panel) was determined by Western analysis of anti-RAD51 immunoprecipitates as above. The Western blot was reprobed for total RAD51 (RAD51; middle panel). BCR/ABL kinase activity (total P-Tyr) was determined in total cell lysates (bottom panel) using anti-P-Tyr antibodies. The results are representative of at least three independent experiments.

rental and BCR/ABL-transformed cells was examined (Figure 6B). RAD51 was immunoprecipitated from parental 32Dcl3 cells and BCR/ABL cells before and after treatment with cisplatin and mitomycin C. Tyrosine phosphorylation of RAD51 was examined by Western analysis using anti-phosphotyrosine-specific antibodies. A significant increase in the tyrosine phosphorylation of RAD51 (P-Tyr-RAD51) was observed in the parental 32Dcl3 cells following treatment with cisplatin and mitomycin C (Figure 6B, compare lanes 3 and 5). This

increase in P-Tyr-RAD51 has been attributed to damage-induced activation of the c-ABL kinase activity (Chen et al., 1999; Yuan et al., 1998). Treatment with cisplatin and mitomycin C produced little if any difference in the tyrosine phosphorylation of RAD51 in BCR/ABL cells (Figure 6B, compare lanes 4 and 6). However, the basal level of RAD51 phosphorylation in BCR/ABL cells appeared to be greater than the drug-induced phosphorylation of RAD51 in parental 32Dcl3 cells (Figure 6B, compare lanes 4 and 5).

STI571 has been shown to be a selective inhibitor of the c-ABL and BCR/ABL kinase (Figure 6C, compare total P-Tyr in lanes 2 and 3; Druker et al., 1996). We found that treatment of BCR/ABL cells with STI571 significantly reduced the level of RAD51 phosphorylation (Figure 6C, compare P-Tyr-RAD51 in lanes 2 and 3) to levels nearly comparable to the parental 32Dcl3 cells (Figure 6C, P-Tyr-RAD51, lane 1). These results suggest that the interaction of BCR/ABL with RAD51 results in the constitutive phosphorylation of RAD51.

BCR/ABL Phosphorylates RAD51 on Tyrosine 315

It has been reported that DNA damage-activated c-ABL phosphorylates RAD51 on Tyr-54 (Yuan et al., 1998) and/or Tyr-315 (Chen et al., 1999). In order to determine which amino acid residue of the RAD51 protein was phosphorylated by the BCR/ABL tyrosine kinase, Flag-tagged RAD51(Y54F) and RAD51(Y315F) substitution mutants were produced by site-directed mutagenesis. COS-7 cells were transiently cotransfected with BCR/ABL (+) or BCLR/ABL(K1172R) kinase mutant (−) and Flag-tagged RAD51, Flag-tagged RAD51 (Y54F), or Flag-tagged RAD51 (Y315F). Flag-tagged RAD51 proteins were immunoprecipitated by anti-Flag antibodies and examined for their phosphorylation status (Figure 7A). No phosphorylation of RAD51 was observed in the presence of BCR/ABL(K1172R) (Figure 7A, lanes 1, 3, and 5). Significant phosphorylation of wild-type RAD51 and RAD51(Y54F) was observed in cells expressing BCR/ABL (Figure 7A, lanes 2 and 6). However, the phosphorylation of the RAD51(Y315F) was dramatically reduced (Figure 7A, lane 4) compared to both the wild-type RAD51 and RAD51(Y54F) (Figure 7A, lanes 2 and 6). It is interesting to note that the RAD51(Y54F) phosphorylation did not appear equivalent to wild-type RAD51, and there was low-level phosphorylation of RAD51(Y315F). These results indicate that the majority of BCR/ABL tyrosine kinase phosphorylation occurs on RAD51(Y315) with a significantly lower level of phosphorylation on hRAD5(Y54) or other tyrosine residues. These conclusions are tempered by the possibility that the presence of a Flag tag on RAD51 may affect the phosphorylation of these residue(s).

To examine the phosphorylation of endogenous RAD51(Y315), antisera was prepared using an unphosphorylated (Y315) and phosphorylated (P-Y315) peptide of RAD51 (Figures 7B and 7C). Consistent with previous results, the Y315 antibody detected elevated levels of RAD51 in BCR/ABL cells compared to the parental 32Dcl3 cells (Figure 7B, compare lanes 1 and 2). Similar results were obtained with the RAD51 whole-protein polyclonal antibody. RAD51 was not detected in the parental 32Dcl3 cells when probed with the P-Y315 phos-

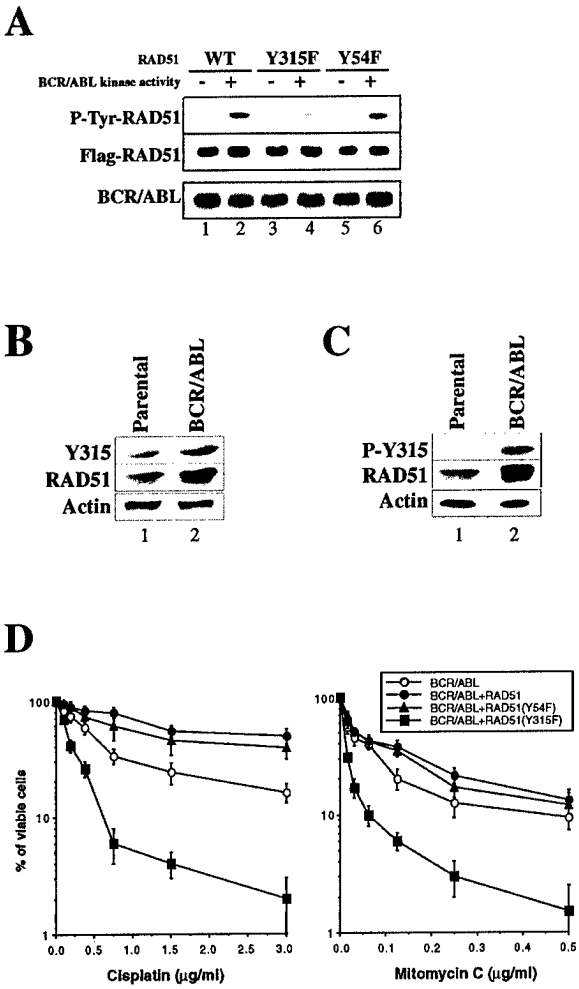

Figure 7. The Role of BCR/ABL-Mediated Tyrosine Phosphorylation of RAD51 in Drug Resistance

(A) Y315F mutation reduced RAD51 phosphorylation by BCR/ABL. Flag-RAD51, Flag-RAD51(Y315F), or Flag-RAD51(Y54F) mutants were cotransfected in COS-7 cells with either BCR/ABL(K1172R) kinase-deficient mutant (−) or with BCR/ABL kinase-active protein (+). The level of RAD51 tyrosine phosphorylation (P-Tyr-RAD51 box) in anti-Flag immunoprecipitates was determined by Western analysis as in Figure 6. The Western blot was reprobed for total Flag-RAD51 with anti-Flag-specific antibodies (middle panel). The expression of BCR/ABL in the transfected cells (bottom panel) was confirmed by Western analysis.

(B and C) BCR/ABL phosphorylates RAD51(Y315). Total cell lysates obtained from 32Dcl3 parental cells (lane 1) and from BCR/ABL cells (lane 2) were examined by Western analysis using specific antibodies recognizing RAD51(Y315) nonphosphorylated ([B], top panel) or RAD51(Y315) phosphorylated ([C], top panel). Anti-RAD51 antibody was used to determine the total level of RAD51 ([B] and [C], middle panel) compared to actin loading control ([B] and [C], bottom panel).

(D) Drug resistance is dependent on RAD51(Y315) phosphorylation. BCR/ABL cells were transduced with pMigR1 containing RAD51, RAD51(Y54F), RAD51(Y315F), or empty vector (IRES-GFP). GFP-positive cells were isolated by FACS and incubated with the indicated concentrations of cisplatin or mitomycin C in the presence of IL-3. After 48 hr, the percentage of viable cells was determined by trypan blue exclusion. The results represent the average and standard deviation of at least three independent experiments.

phospecific antibody (Figure 7C, lane 1), even though RAD51 was easily detected with the RAD51 whole-protein polyclonal antibody. In contrast, a significant P-Y315 signal was observed in cells expressing BCR/ABL (Figure 7C, lane 2).

Phosphorylation of RAD51(Y315) Controls Resistance to Cisplatin and Mitomycin C

To determine the effect of RAD51 phosphorylation status on drug resistance, we transduced BCR/ABL cells with MigR1-GFP retroviral vector containing wild-type RAD51, RAD51(Y54F), or RAD51(Y315F). GFP-positive cells were subsequently isolated by FACS. Following treatment with cisplatin and mitomycin C, the percentage of viable cells was determined by trypan blue exclusion (Figure 7D). The results suggest that transduction of wild-type RAD51 or RAD51(Y54F) into BCR/ABL cells did not affect their resistance to cisplatin or mitomycin C (Figure 7D). Indeed, both the wild-type RAD51 and RAD51(Y54F) appeared to modestly enhance drug resistance of BCR/ABL cells, presumably by further increasing the levels of cellular RAD51. However, transduction of RAD51(Y315F) decreased cisplatin and mitomycin C drug resistance (Figure 7D) to the drug-sensitivity levels observed with BCR/ABLΔΔ cells (Figure 1B and data not shown). In addition, RAD51(Y315F)-dependent reduction of drug resistance occurred in the presence of endogenous wild-type RAD51, suggesting that the transduced mutant is dominant.

Discussion

The Philadelphia chromosome, Ph1, results in the BCR/ABL translocation that appears to constitutively activate the ABL kinase (Sawyers, 1999). Leukemia cells containing the BCR/ABL tyrosine kinase have been shown to be resistant to a number of therapeutic drugs as well as γ irradiation (Amarante-Mendes et al., 1998; Bedi et al., 1995; Dubrez et al., 1998). The mechanism(s) of BCR/ABL-mediated drug resistance has not been well characterized. While the inhibition of caspase-3 activation and induction of a G2/M cell cycle arrest may play significant roles, other cellular functions may be equally responsible for therapeutic drug resistance.

BCR/ABL-Mediated Regulation of Rad51-Related Proteins Enhances DSB Repair and Induces Drug Resistance

In general, there appears to be a fundamental correlation between the expression of RAD51 and resistance to therapeutic drugs (Vispe et al., 1998). Elevated levels of RAD51 have also been linked to chlorambucil resistance in B cell chronic lymphocytic leukemia (Christodoulopoulos et al., 1999) and with enhanced survival of pancreatic adenocarcinoma after treatment with the DSB-inducing therapeutic drug calicheamicin γ1 (Maacke et al., 2000). Conversely, downregulation of RAD51 increased the radiosensitivity of prostate cancer cells (Collis et al., 2001) and malignant glioma cells (Ohnishi et al., 1998). Here we have shown that the BCR/ABL oncogenic tyrosine kinase induces the expression of RAD51 and several related RAD51-Paralog proteins. The high levels of RAD51 result from both elevated STAT5-dependent transactivation of the RAD51 gene and the reduction of RAD51 protein cleavage by caspase-3. It is possible that the regulation of other RAD51-Paralog members occurs by a similar mechanism.

Recombination repair has been associated with the processing of both cisplatin and mitomycin C DNA lesions (Bhattacharyya et al., 2000). Our studies provide a link between elevated expression of RAD51-Paralog members, enhanced homologous recombination, and resistance to cisplatin and mitomycin C in BCR/ABL cells. Reciprocally, the downregulation of RAD51 decreased the drug resistance and homologous recombination in BCR/ABL cells. These results strongly implicate enhanced RAD51-Paralog protein-dependent recombination repair as an important mechanism for the cisplatin and mitomycin C drug resistance observed in BCR/ABL tumor cells. A role for RAD51-Paralogs in therapeutic drug resistance appears specific for drugs which induce DSBs, since elevated levels of RAD51 did not affect sensitivity to MNNG.

RAD51 has also been suggested to play an essential role in the repair of chromosome breaks in normal proliferating cells (Sonoda et al., 1998). Thus, a "basal" level of RAD51 appears to be required for the processing of spontaneous DNA lesions. Oncogenes that alter the expression, phosphorylation, nuclear localization, and/or function of RAD51 may alter global DNA repair efficiency and lead to drug resistance (and perhaps selection) of neoplastic or preneoplastic cells. In addition, dysregulated RAD51-dependent homologous recombination may contribute to genomic instability by compromising the fidelity of HR (for review see Bishop and Schiestl, 2001), by altering the balance between HR and other recombination repair pathways (Richardson and Jasin, 2000), or by altering the checkpoint processes associated with DNA repair. Thus, even modest changes in the cellular levels of the RAD51-Paralog proteins may significantly promote drug resistance, genomic instability, and perhaps tumorigenesis.

BCR/ABL Phosphorylates RAD51

We found that a portion of BCR/ABL and RAD51 form a perinuclear and/or cytoplasmic complex that results in the strong constitutive phosphorylation of RAD51(Y315) but only modest phosphorylation of RAD51(Y54). The RAD51(Y315F) but not the RAD51(Y54F) mutant decreased BCR/ABL-dependent drug resistance, suggesting that the unique phosphorylation of RAD51(Y315) has a substantial impact on DNA repair. It is interesting to note that the RAD51(Y315) residue is present in a YXXP context, a sequence preferred by the c-ABL kinase (Andoniou et al., 1996). This RAD51 motif is highly conserved in all known species of the animal kingdom.

The functional role of RAD51(Y315) phosphorylation is unknown. We have observed alterations of both nuclear localization and nuclear foci formation associated with the mutation of RAD51(Y315) (data not shown). Since RAD51 is known to interact with other proteins, such as BRCA1, BRCA2, XRCC3, and/or RAD52, it is possible that appropriate phosphorylation of RAD51(Y315) is required for the assembly of nuclear foci in BCR/ABL tumor cells (Bhattacharyya et al., 2000; Bishop et al., 1998; Haaf et al., 1999; Yuan et al., 1999). In support of this

idea, the phosphorylation of RAD51 on Y315 (but not Y54, Y205, or Y191) by activated c-ABL has been shown to enhance complex formation between RAD51 and RAD52 (Chen et al., 1999), which would presumably stimulate homologous pairing (Benson et al., 1998). Further studies will be required to determine the role, if any, of RAD51(Y315) phosphorylation.

Taken as a whole, our studies suggest that BCR/ABL (and perhaps other oncogenic tyrosine kinases) modulates the expression of RAD51-Paralog proteins and the phosphorylation of RAD51. The increase in quantity of RAD51-Paralog members results in an enhanced response to DNA damage. This augmented response to DNA damage contributes significantly to the resistance of crosslinking and DSB-inducing agents in BCR/ABL leukemia cells.

Experimental Procedures

Plasmids

See Supplemental Table S1 at http://www.molecule.org/cgi/content/full/8/4/795/DC1.

Cells

Bone marrow cells from CML-chronic phase and CML-blast crisis patients and from normal healthy volunteers were obtained after informed consent. Nonadherent and T cell-depleted bone marrow cells (A^-T^- BMC) and $CD34^+$ cells were isolated as described (Skorski et al., 1995) and cultured in IMDM supplemented with 10% FBS and recombinant human IL-3 (when indicated). Murine growth factor-dependent parental 32Dcl3 myeloid cells as well as their BCR/ABL-transformed counterparts (Nieborowska-Skorska et al., 1999) were cultured in IMDM supplemented with 10% FBS and IL-3. Parental 32Dcl3 cells expressing BCR/ABL tsp210 or human RAD51 (hRAD51) were generated by electroporation with the plasmid encoding BCR/ABL tsp210 or hRAD51 gene followed by puromycin selection of single clones. Expression of BCR/ABL and hRAD51 was confirmed by Western analysis. Parental 32Dcl3 cells expressing other BCR/ABL proteins and STAT5B mutants have been described (Nieborowska-Skorska et al., 1999). Tk^-ts13 hamster fibroblasts and COS-7 green monkey kidney cells were cultured in DMEM or RPMI, respectively, supplemented with 10% FBS. Draa cells (Pierce et al., 1999) were cultured in α-MEM supplemented with 10% FBS.

Drug-Resistance Assays

Indicated concentrations of cisplatin (Platinol-AQ; Bristol-Myers Squibb Co., Princenton, NY) or mitomycin C (Sigma Chemical Co., St. Louis, MO) were added to cells growing in suspension (10^5/ml) or in semisolid medium (10^3/ml) (MethoCult H4230; StemCell Technologies Inc., Vancouver, B.C.) supplemented with IL-3. Viable cells in suspension cultures were detected 48 hr later by trypan blue exclusion. Apoptotic cells were detected on cytospin slides by TACS in situ apoptosis detection kit (Trevigen, San Diego, CA). Colonies growing in semisolid medium were counted after 7 days.

Apoptosis Induced by Growth Factor Withdrawal

Cells (10^5/ml) were starved from IL-3 for 24 and 48 hr. Apoptotic cells were detected on cytospin slides by TACS in situ apoptosis detection kit (Trevigen, San Diego, CA).

Northern Analysis

32Dcl3 cells expressing BCR/ABL and/or STAT5B or STAT5B-DAM proteins were starved from growth factor for 12 hr. Parental 32Dcl3 cells were starved or not starved from IL-3 as indicated. Total RNA (10 μg) was isolated and analyzed for the expression of RAD51 by Northern analysis probed with a 177 bp fragment of murine RAD51 end-labeled with [α-^{32}P]dCTP (NEN Life Science Products, Inc., Boston, MA).

Immunoprecipitation and Western Analysis

Cells were lysed in EBC buffer plus protease inhibitors (Chen et al., 1999). Immunoprecipitates and cell lysates were resolved on SDS-PAGE and examined by Western analysis using the following antibodies: anti-ABL (Ab-3; Oncogene Research Products, Cambridge, MA), anti-RAD51 (C20; Santa Cruz, Santa Cruz, CA), anti-P-Tyr (PY20 from Oncogene Research Products and 4G10 from Upstate Biotechnology, Lake Placid, NY), anti-actin (C11; Santa Cruz), rabbit anti-caspase-3 (CM1; generous gift of IDUN Pharmaceuticals, Inc., La Jolla, CA). Polyclonal rabbit antibodies were raised against hRAD51(Y315) phosphorylated (P-Y315) peptide and nonphosphorylated (Y315) peptide containing the peptide sequence ETRICKIY*D SPCLPEAEAM (the asterisk indicates Y315; Novus Biologicals, Littleton, CO).

Luciferase Assay

Tk^-ts13 cells were transiently transfected by calcium phosphate (Skorski et al., 1997) with plasmids encoding BCR/ABL wild-type or mutants and/or STAT5B, PI-3k, Akt, c-Myc wild-type or mutants, as well as the RAD51 reporter plasmid. The reporter plasmid fused the CpG-rich region of the human RAD51 promoter (Schmutte et al., 1999), the SV40 promoter, and the luciferase gene (RAD51-SV40-luc). The negative control plasmid encoded the SV40 promoter fused to the luciferase gene only (SV40-luc). A β-galactosidase plasmid was also transfected into the Tk-ts13 cells as a transfection efficiency control. When BCR/ABL was cotransfected with the dominant-negative mutants, a total of 20 μg of DNA/10 cm plate was used (BCR/ABL:DNM ratio = 1:4). Twenty-four hours after transfection, serum-free medium containing 0.1% BSA was added to the cells. Thirty-six hours later, luciferase was quantified by the Luciferase Assay System (Promega, Madison, WI). Transfection efficiency was normalized by measuring β-galactosidase activity. Transactivation units were calculated as a ratio of the counts from RAD51-SV40-luc to the counts from SV40-luc in particular groups.

Inhibition of Caspase-3

Parental 32Dcl3 cells and BCR/ABL cells or BCR/ABLΔΔ cells were untreated or treated (as indicated) with cisplatin (1 μg/ml) for 24 hr in the presence of IL-3. Caspase-3 inhibitor Z-DEVD-fmk (50 μM; Calbiochem-Novabiochem Co., San Diego, CA) was added when indicated.

GFP-Positive Cells

Cells were transduced with retroviral vector particles encoding GFP as described (Nieborowska-Skorska et al., 1999). Transduced cells were collected after 48–72 hr of cocultivation with the packaging cell line. GFP-positive cells were isolated by FACS.

Expression of RAD51-Flag Proteins

COS-7 cells were transfected with BCR/ABL or the BCR/ABL (K1172R) mutant and with the Flag-tagged RAD51, the Y315F mutant, or the Y54F mutant by calcium phosphate as described (Skorski et al., 1997). Forty-eight hours after transfection, serum-free medium containing 0.1% BSA was added to the cells for the next 12 hr.

Inhibition of BCR/ABL Kinase

ABL kinase inhibitor STI571 (Druker et al., 1996) was obtained from NOVARTIS Pharma AG (Basel, Switzerland). Cells (10^6/ml) were incubated for 24 hr with 1 μM of the inhibitor in the presence of IL-3, then washed and used for experiments.

Immunofluorescence

Cytospin slides were prepared from cells grown in the presence of IL-3. Cells were permeabilized with cold acetone. RAD51 was detected by mouse anti-RAD51 monoclonal antibody (UBI, Lake Placid, NY) followed by TRITC-conjugated goat anti-mouse secondary antibody (Molecular Probes). BCR/ABL was detected with rabbit anti-BCR/ABL antibody (protein A-purified immunoglobulins from the rabbit immunized with 12 aa peptide corresponding to the BCR/ABL b3/a2 junction region), which recognizes BCR/ABL but not c-ABL (T.S., unpublished data), followed by AlexaFluor488-conjugated goat anti-rabbit secondary antibody (Molecular Probes). Negative controls were performed without primary antibodies. DNA was

counterstained with the DNA fluorochrome 4′,6′diamedino-2-phenylindole (DAPI). Cells were visualized with a Nikon Eclipse E300 fluorescence microscope equipped with a digital camera. Images were prepared with Adobe Photoshop.

DSB Repair Assay

Draa cells (generously provided by Dr. M. Jasin, Sloan-Kettering Cancer Center, New York, NY) have integrated one or two copies of the modified gene for GFP (SceGFP) as a recombination reporter and a fragment of the GFP gene as a donor for homologous repair (Pierce et al., 1999). SceGFP has an inactivating insertion containing the restriction site for the rare-cutting I-SceI endonuclease. When I-SceI is expressed in vivo, a DSB results. A homologous repair event with a donor GFP gene fragment restores functional GFP expression, readily detected by flow cytometry. Draa clones expressing BCR/ABL wild-type, BCR/ABL K1172R kinase-deficient mutant, and control clones expressing a *neo*-resistance gene only were established. Cells were electroporated as described (Pierce et al., 1999) with 100 μg I-SceI plasmid and 40 μg RAD51 antisense plasmid or empty plasmid. Forty-eight hours later, 5×10^4 cells were analyzed by flow cytometry for the presence of GFP. The presence of BCR/ABL proteins did not affect the transfection efficiency of Draa cells or the ability of I-SceI to induce double-strand breaks in the SceGFP sequence.

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